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Molecular Stability of Chicken and Rabbit Immunoglobulin G

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Molecular stability of chicken egg yolk immunoglobulin G (IgY) and that of rabbit IgG were compared by measuring antibody activities and conformational changes. Stability of rabbit IgG to acid denaturation was much higher than that of IgY. Conformation of the IgY molecule was readily changed in acidic conditions, resulting in a rapid loss of antibody activity. Much less stable natures of IgY to heat-treatment and guanidine-HCl denaturation than rabbit IgG were also observed. Differences in the structure between the two immunoglobulins that might participate in their different stability were inferred from their amino acid sequence data. Importance of the intramolecular disulfide linkage in the rabbit light chain and some other structural differences were suggested.

Immunoglobulin G antibodies in chicken blood serum are efficiently transferred across the follicular epithelium of the ovary and accumulated in the yolk during oogenesis.[1] More than 100 mg of chicken egg yolk immunoglobulin (called IgY[2]) can be isolated from one egg, indicating that at least 30 g of antibody can be obtained in one year by immunizing only one chiken without any burdensome labor such as bleeding. Chicken egg yolk has been, therefore, taken much attention as a good and convenient source of antibodies. IgY has many characteristics different from IgG of mammalian origin. For example, IgY does not bind with mammalian complements[3] nor Rheumatoid factor[4] which are present in serum from mammalian species. Anti-mammalian IgG antibodies, which often cross-react to IgG from different mammalian species, show a poor cross-reactivity to IgY.[5] These characteristics are advantageous when IgY antibodies are used as biochemical/clinical reagents.

Although IgY is classified as an IgG-class immunoglobulin, the structure of IgY is considerably different from mammalian IgG. The larger molecular weight of the heavy chain (68,000 for IgY *vs.* 50,000 for mammalian IgG) is one example of the differences.[2] Recently, cloning and sequencing of genes encoding the heavy[6,7] and light[8,9] chains of IgY were done, and the primary structure of these polypeptides has been described. However, structural properties of IgY, especially the stability of the IgY molecule, in comparison with those of mammalian IgG have not yet been fully investigated, although the stability of IgY and bovine IgG has been partially compared by Shimizu *et al.*[10] Knowing the molecular stability of IgY is particularly important when IgY is used as a reagent under various conditions. In this study, the stability of IgY has been investigated and compared with that of rabbit serum IgG, which is quite frequently used as an immunochemical reagent. Structural characteristics of the two immunoglobulins were also inferred from their amino acid sequence data and their possible effects on the molecular stability were discussed.

Materials and Methods

Antibodies. Specific egg yolk antibodies (IgY) were obtained from White Leghorn hens immunized with such antigens as mouse IgG, bovine serum albumin (BSA), and rotavirus-Wa strain. Immunization was started by intramuscularly injecting hens with 1 mg (mouse IgG or BSA) or 10^7 FCFU (rotavirus) antigens in 1 ml phosphate-buffered saline (PBS) emulsified with an equal volume of complete Freund's adjuvant. Booster shots were also given intramuscularly at 2-week intervals after the first injection with the same dose. Egg samples were collected and IgY was purified from the egg yolk by the procedure described by Hatta *et al.*[11] or by Polson *et al.*[12] Specific rabbit antibodies (IgG) were obtained from New Zealand white rabbits immunized with the same antigens. The immunization method is same as that used for chicken antibody production. Blood serum samples were collected 1 week after the fourth immunization and IgG was purified from the sera by Protein A-affinity chromatography (MAPS-II kit: Bio-Rad Laboratories). Non-specific IgY and rabbit IgG antibodies were isolated and purified from egg yolks of non-immunized chickens and from normal rabbit sera, respectively, by the methods used for the specific antibody purification described above.

Enzyme-linked immunosorbent assay (ELISA). Measurement of the antibody activity of the purified IgY or IgG antibodies was done by ELISA. Anti-mouse IgG and anti-BSA antibody activity was measured by indirect ELISA. The indirect ELISA was done as described previously,[10] except that the polystyrene microtiter plate was coated with mouse IgG or BSA. Anti-rotavirus antibody activity was measured by sandwich ELISA as described by Ebina *et al.*[13]

Acid and alkali treatment. An antibody solution (20 mg/ml 25 mM phosphate buffer, pH 7.0) was diluted with PBS the pH of which had been adjusted with HCl or NaOH to make the final pH of the solution 2—7 or 11—13. The solution (final protein concentration of 1 mg/ml) was incubated at 37°C for 0—7 hr. After incubation, the solution was neutralized by 1000-fold dilution with PBS containing 0.05% Tween-20 (PBS-Tw), and the antibody activity was measured by ELISA.

Heat treatment. An antibody solution (1 mg/ml PBS) was heated for 30 min (at 62.5°C) or 15 min (at 65°C or higher). The antibody activity of the heated samples was measured by ELISA.

Fluorescence measurement. An immunoglobulin solution (protein concentration 0.02—0.05%) was excited at 296 nm and the wave-length at which the emission fluorescence was maximal was measured by a Hitachi F-4000 fluorescence spectrophotometer. The wavelength for the emission maximum was plotted against pH or the concentration of GuHCl.

Circular dichroism. The CD spectra were taken at 25°C with a spec-

tropolarimeter (Jasco, Model J-600). A quartz cell with 0.2-cm path length was used. The protein sample was dissolved in 50 mM phosphate buffer adjusted to pH 2, 4, or 7.2 to give the final protein concentration of 0.01%. After incubating the solution at 37°C for 3 hr, CD measurements were done. The results are expressed as mean residue ellipticity (θ), which is defined as $\theta] = (100 \times \theta_{obs})/(l \times c)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles/liter, and l is the lightpath length (cm). A value of 108 was used as the mean residue molecular weight.

Prediction of the conformational properties from the primary structure. The secondary structures were predicted for each domain of IgY and rabbit IgG by the method of Chou and Fasman.[14] The chain flexibility was predicted for each domain or framework region by the method of Karplus and Schulz.[15] The sequence data for the rabbit L chain (κ-chain) were from Kindt[16] and Lieberman *et al.*[17] The sequence data for heterogeneous rabbit γ-chains were from Hill *et al.*[18] and Dayhoff,[19] and those for the rabbit VH region were deduced from the sequence of nucleotide sequence of a rabbit VH region gene reported by Gallarda *et al.*[20] The sequence data for chicken L chain were from the nucleotide sequence of a λ-chain gene reported by Reynaud *et al.*[5] The sequence data for chicken H chain, including the VH region, were from Reynaud *et al.*[7] and Parvari *et al.*[6]

Results

Stability of immunoglobulins at acidic conditions

Changes in the activity of anti-mouse IgG IgY and rabbit IgG antibodies after incubating under acidic conditions were measured by ELISA and the results are shown in Fig. 1. The activity of IgY antibodies was decreased by incubating at pH 3.5 or lower and almost completely lost at pH 3. On the other hand, the activity of rabbit IgG antibodies did not change significantly until the pH of the solution was decreased to 2.0. Even at pH 2, the decrease in the ELISA value was less than 20%. Anti-BSA and anti-rotavirus antibodies produced in chickens and rabbits also showed patterns similar to those observed for anti-mouse IgG antibodies.

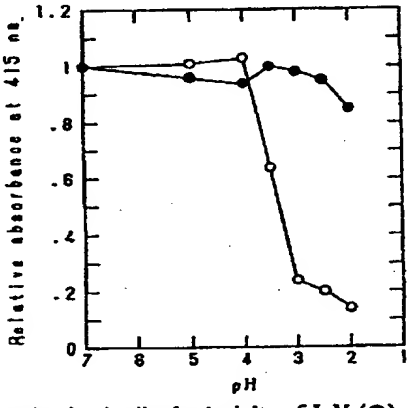

Fig. 1. Changes in the Antibody Activity of IgY (○) and Rabbit IgG (●) after Incubating at Acidic Conditions.

Incubation was done at 37°C for 7 hr. The antibody (anti-mouse IgG) activity was measured by ELISA and expressed as the relative absorbance at 415 nm.

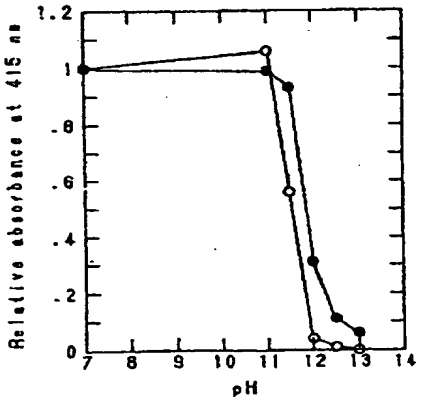

Fig. 2. Changes in the Antibody Activity of IgY (○) and Rabbit IgG (●) after Incubating at Alkaline Conditions.

Incubation was done at 37°C for 7 hr. The antibody (anti-mouse IgG) activity was measured by ELISA.

Stability of immunoglobulins at alkaline conditions

IgY and rabbit IgG antibodies were incubated under alkaline conditions (pH 11—13) and their activity was measured by ELISA. Changes in the activity of anti-mouse IgG antibodies after incubation are shown in Fig. 2. The activity did not change until the pH increased to 11 but was markedly diminished by incubating at pH 12 or higher. These changes were similarly observed for both of the antibodies, and for antibodies of different specificities.

Heat stability of immunoglobulins

IgY and rabbit IgG antibodies were heated at 62.5°C or higher for 15—30 min and their activity was measured by ELISA. As shown in Fig. 3, the activity of IgY antibodies

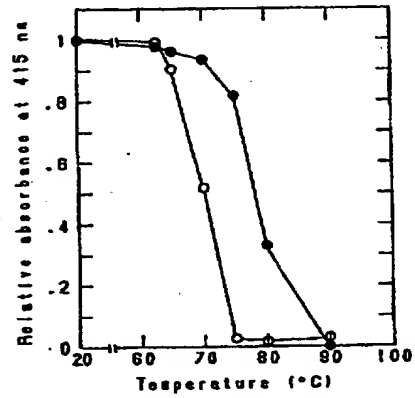

Fig. 3. Changes in the Antibody Activity of IgY (○) and Rabbit IgG (●) after Heating for 30 min (at 62.5°C) or 15 min (at 65°C or Higher).

The antibody (anti-mouse IgG) activity was measured by ELISA.

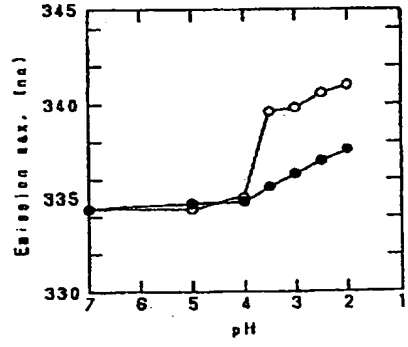

Fig. 4. Denaturation Curves of IgY (○) and Rabbit IgG (●) at Acidic Conditions.

Denaturation was evaluated by measuring the wavelength at which the emission fluorescence was maximal. Non-specific immunoglobulin samples were used for the measurement after incubation for 3 hr.

was decreased by heating for 15 min at 70°C or higher, while that of rabbit IgG antibodies decreased at 75—80°C or higher.

Conformation changes of immunoglobulins under acidic conditions

The degree of conformation changes in immunoglobulin molecules under acidic conditions was evaluated by measuring fluorescence changes of the internal tryptophan residues. As shown in Fig. 4, the marked wavelength shift of Em_{max} for IgY occurred between pH 4 and 3.5, while the wavelength shift for rabbit IgG at pH 4 or below was gradual and very small. These tendencies were similar to those obtained by ELISA (Fig. 1). Changes in the secondary structures of IgY and rabbit IgG were investigated by measuring CD patterns of these proteins at acidic pHs. The CD pattern of IgY at pH 2 (Fig. 5) indicated that the secondary structure of IgY was destroyed at this pH. In contrast, destruction of the secondary structure of rabbit IgG at pH 2 was smaller, indicating that the secondary structure in the rabbit IgG molecule is more stable under acidic conditions than that in the IgY molecule.

Conformation changes of immunoglobulins in a guanidine–HCl solution

The degree of conformation changes in immunoglobulin molecules occurring in a GuHCl solution was also evaluated by a fluorescence spectroscopic method. Figure 6 indicates the effects of GuHCl concentration on the conformation changes in the IgY and rabbit IgG molecules. Conformation changes of IgY started in 2.5—3 M GuHCl and almost finished in 3.5 M GuHCl, indicating that the conformation change rapidly occurred in a very narrow range of GuHCl concentration. On the other hand, the conformation change of rabbit IgG started in 3—3.5 M GuHCl and gradually proceeded. A complete change of rabbit IgG did not occur even in 5 M GuHCl.

Prediction of the conformational properties of immunoglobulins from their primary structure

The structures of IgY and rabbit IgG are compared in Fig. 7, and the major structural differences between the two immunoglobulins are summarized in Table I.

The secondary structures in the constant domains were predicted and the results are shown in Table II. The content of the β-sheet structure, which is known to be the

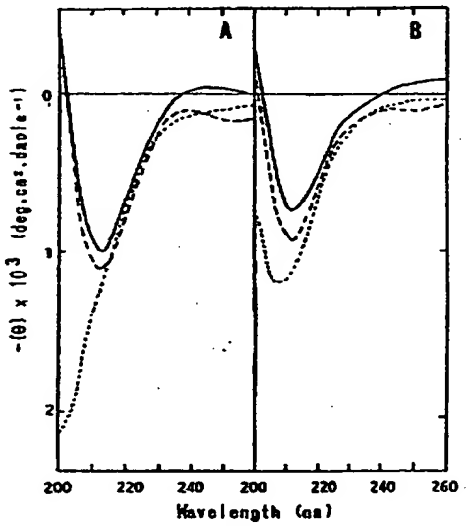

Fig. 5. Circular Dichroism of IgY (A) and Rabbit IgG (B) at Acidic Conditions.

——, at pH 7.2; - - - -, at pH 4; ·····, at pH 2. Non-specific immunoglobulin samples were used for the measurement.

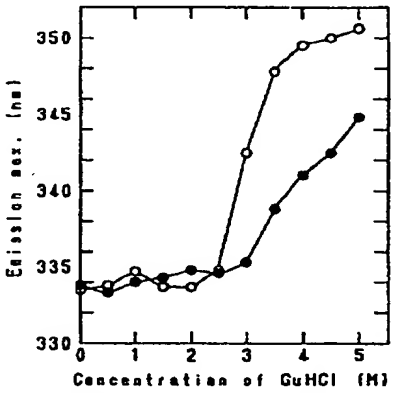

Fig. 6. Guanidine–HCl Denaturation Curves of IgY (○) and Rabbit IgG (●).

Non-specific immunoglobulin samples were used for the measurement.

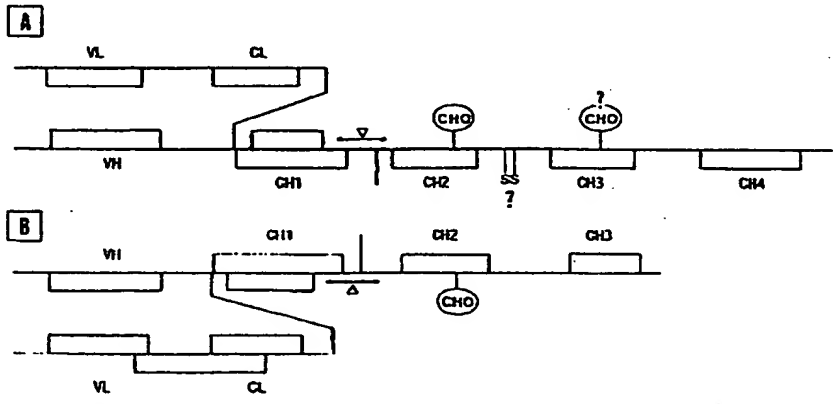

Fig. 7. Comparison of the Structure between IgY (A) and Rabbit IgG (B).

Illustrations were made according to the primary sequence data reported previously [6-9,16-20] CHO, carbohydrate chain; ▽, the boundary region (30 residues) between CH1 and CH2, which includes the hinge region (for rabbit IgG, residues 215—229).

Table I. Major Structural Differences between IgY and Rabbit IgG Deduced from Their Amino Acid Sequences

	IgY	Rabbit IgG
Number of amino acid residues	H-chain 559 L-chain 206	H-chain 440 L-chain 214
Number of CH domains	4	3
Intermolecular S–S between H–H	Cys243 Cys331 (?) Cys338 (?)	Cys224
Intramolecular S–S in the L-chain	Cys20–Cys83 Cys131–Cys188	Cys23–Cys88 Cys134–Cys194 Cys80–Cys171
N-Glycosylation site in the H-chain	–Asn(299)–Val–Ser– –Asn(398)–Gly–Thr–(?)	—Asp(291)–Ser–Thr–

Table II. Prediction of the Secondary Structures in the IgY and Rabbit IgG Domains

Domains	IgY		Rabbit IgG	
	α-Helix (%)	β-Sheet (%)	α-Helix (%)	β-Sheet (%)
CH1	29.5	24.8	5.4	40.2
2	18.1	21.3	29.2	34.9
3	17.4	38.5	21.0	31.4
4	33.9	36.4	—	—
CL	14.7	33.3	4.3	47.4

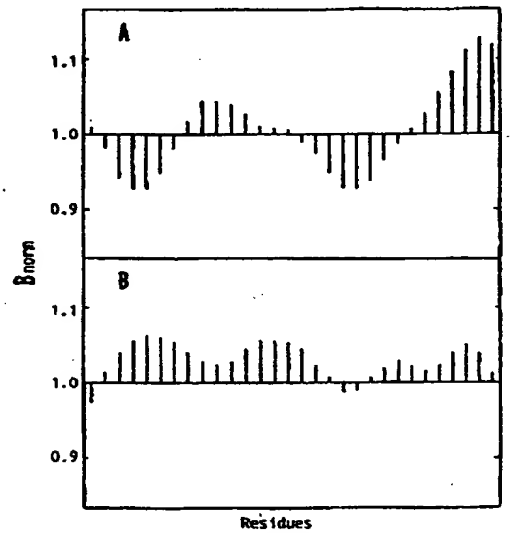

Fig. 8. Chain Flexibility Profiles for the Boundary Region between CH1 and CH2 of IgY (A) and Rabbit IgG (B).

The chain flexibility (B_{norm} value) was predicted for the boundary regions (residues 219—248 for IgY, residues 205—234 for rabbit IgG) shown by ▽ in Fig. 7. The B_{norm} value higher than 1.0 means "flexible," while the value lower than 1.0 means "rigid."

predominant, important secondary structure of immunoglobulins, was predicted to be higher in rabbit IgG. The chain flexibility profiles of each domain were similar between the two immunoglobulins; especially those for the framework regions (data not shown). However, the flexibility profiles for the regions corresponding to the hinge of the H chain (see Fig. 7) were predicted to be more flexible for rabbit IgG than for IgY, as shown in Fig. 8.

Discussion

Different stability profiles of chicken IgY and rabbit IgG were observed in this study. Although the stability of these immunoglobulins in alkaline conditions was similar, rabbit IgG showed higher stability to acid denaturation than chicken IgY (Fig. 1). Similar stability differences have been also observed when the activity of anti-rotavirus antibodies after acid-treatment was measured by the virus-neutralization test (data not shown). Rapid decrease of the IgY antibody activity in these assays indicates that damage in the antigen-binding site of IgY were caused by incubating under acidic conditions, while that of rabbit IgG did not. Since the reactivity of anti-[IgY-Fab] antibody to IgY has been observed to be decreased by acid-treatment of IgY (Lee *et al.*, unpublished results), conformational changes of the Fab portion including the antigen-binding site must happen during acid treatment.

The conformation changes in the IgY molecule under acid conditions were confirmed by fluorescence study (Fig. 4). Since changes in the CD pattern of IgY under acidic conditions were also marked (Fig. 5), destruction of the secondary structure was considered to proceed readily in the IgY molecule when incubated under acidic conditions. As to the rabbit IgG molecule, however, these changes were much smaller, suggesting that the conformation of rabbit IgG in acidic conditions is more rigid and stable. The conformation changes of IgY occurring in acidic conditions were observed to be irreversible when incubation was done below pH 3.5 (data not shown).

Heat-denaturation (Fig. 3) and GuHCl-denaturation experiments (Fig. 6) also showed that the conformation of rabbit IgG molecules was less changeable than that of IgY. Although the mechanisms of conformation change or denaturation of proteins by acid, heat, and such denaturants as GuHCl are though to be different, these results demonstrated that the conformation stability of rabbit IgG was higher than that of IgY in any type of treatment used here, suggesting that the overall stability of the rabbit IgG molecule was higher than that of the IgY molecule. Dreesman and Benedict[21] suggested that the intermolecular noncovalent bondings between the reduced heavy (H) and light (L) chains of IgY were weaker than those of the rabbit IgG chains, since the H and L chains of IgY after reduction were dissociated more easily than those of rabbit IgG. This study has suggested that not only the non-covalent interaction between the H and L chains but also the intramolecular forces supporting the protein conformation are weaker in IgY than in rabbit IgG.

The structural factors that contribute to the different stability of these two immunoglobulins are unknown, because immunoglobulins are large complicated molecules with heterogeneous polypeptides, H and L chains, with multiple domains, the conformation of each domain being changed independently.[22] Since there is no experimental information on the structural properties of the IgY domains, we have tried to estimate them from their amino acid sequences reported earlier.[6-9,16-20]

The predicted values for the secondary structure were different between IgY and rabbit IgG, the lower content of the ordered structure, especially the β-sheet, in most of the IgY domains being observed (Table II). It is well known that the structure, called "basic immunoglobulin fold" packed between the two β-sheet structures formed by several β-strands, is common to all mammalian immunoglobulin domains.[22,23] Although the prediction by the method of Chou and Fasman[14] is considered to be not always correct, the lower content of the β-structure in IgY may indicate that the conformation of the IgY domains is more disordered and less stable than that of the rabbit IgG domains.

One peculiar difference between IgY and rabbit IgG deduced from their sequences is the presence of an intrachain disulfide linkage between the VL and CL domains of rabbit κ-chain which occupies 95% of the domestic rabbit L-chain[24] (Fig. 7). This linkage is likely to stabilize the structure of the rabbit L-chain and subsequently slow down the inactivation of rabbit IgG by acid or denaturant. Since this type of intrachain disulfide linkage is not present in the IgY L-chain, this may be one of the major causes of the higher stability of rabbit IgG. Importance of the intrachain disulfide linkage in stabilization of CL domain was studied in detail by Ashikari *et al.*[25] They have reported that the stability of the native CL domain to acid was 100 times higher than that of a reduced CL domain.

From the primary structure data, other differences were also observed between IgY and rabbit IgG. Unlike rabbit IgG, IgY has two additional Cys residues, Cys331 and Cys338 (Fig. 7), in the CH2-CH3 junction. Since free SH residues were not significantly detected in IgY (data not shown), these Cys residues are likely to participate in the inter-H chain disulfide linkages. In contrast to mammalian IgG, which has a glycosylation site only in the CH2 domain, IgY was observed to have another possible glycosylation site in the CH3 domain (Fig. 7). These structures suggest that IgY resembles such mammalian immunoglobulin classes as IgE,[23] rather than IgG, and they possibly affect the properties of IgY. The lack of a hinge region could be another factor affecting the molecular stability of IgY. The rabbit IgG hinge region was estimated to be flexible by calculating the chain flexibility, while the boundary region between the CH1 and CH2 domains of IgY, which corresponds to the mammalian hinge region, was estimated to be less flexible (Fig. 8). The lower flexibility of this portion may cause the rapid inactivation of the IgY antibody by various treatments, because the flexibility of the hinge region is considered to influence the overall properties of the immunoglobulin molecule as concluded by Pilz *et al.*[26]

The mechanism of the pH-induced conformational changes of immunoglobulins has been an important problem to be solved. But the immunochemists have left the answers incomplete for more than two decades.[22] The detailed analyses of the structure and properties of IgY, which seems to have a unique molecular stability, may give valuable information for solving this problem.

During the preparation of this manuscript, comparative studies on the properties of IgY and rabbit IgG have been reported by Ohtani *et al.*[27] By using anti-α_{s1}-casein antibodies, they found that IgY was more susceptible to protease digestion, although the stability of rabbit IgG observed in their study was only slightly higher than that of IgY.

References

1) M. E. Rose and E. Orlans, *Develop. Comp. Immunol.*, 5, 15—20 (1981).
2) G. A. Leslie and L. W. Clem, *J. Exp. Med.*, 130, 1337—1352 (1969).
3) P. S. Gardner and S. Kaye, *J. Virol. Med.*, 4, 257—262 (1982).
4) A. Larsson and J. Sjoquist, *J. Immunol. Methods*, 108, 205—208 (1988).
5) K. May, S. J. Senior, and P. Porter, in "Immunological Aspects of Reproduction in Mammals," ed. by D. B. Crighton, Butterworths, London, 1984, pp. 13—25.
6) R. Parvari, A. Avivi, F. Lentner, E. Ziv, S. Tel-Or, Y. Burstein, and I. Schechter, *EMBO J.*, 7, 739—744 (1988).
7) C.-A. Reynaud, A. Dahan, V. Anquez, and J.-C. Weill, *Cell*, 59, 171—183 (1989).
8) C.-A. Reynaud, A. Dahan, and J.-C. Weill, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 4099—4103 (1983).
9) R. Parvari, E. Ziv, E. Lentner, S. Tel-Or, Y. Burstein, and I. Schehter, *EMBO J.*, 6, 97—102 (1987).
10) M. Shimizu, R. C. Fitzsimmons, and S. Nakai, *J. Food Sci.*, 53, 1360—1366 (1988).
11) H. Hatta, M. Kim, and T. Yamamoto, *Agric. Biol. Chem.*, 54, 2531—2535 (1990).
12) A. Polson and M. B. Von Wechmar, *Immunol. Commun.*, 9, 475—493 (1980).
13) T. Ebina, K. Tsukada, K. Umezu, M. Nose, K. Tsuda, H. Hatta, M. Kim, and T. Yamamoto, *Microbiol. Immunol.*, 34, 617—629 (1990).
14) P. Y. Chou and G. D. Fasman, *Adv. Enzymol.*, 47, 45—148 (1978).
15) P. A. Karplus and G. E. Schulz, *Naturwissenschaften*, 72, 212—213 (1985).
16) T. J. Kindt, *Adv. Immunol.*, 21, 35—86 (1975).
17) R. Lieberman, L. Emorine, and E. E. Max, *J. Immunol.*, 133, 2753—2756 (1984).
18) R. L. Hill, H. E. Fellows, Jr., and R. Delaney, in "Gamma Globulins: Structure and Control of Biosynthesis," ed. by J. Killander, Interscience, New York, 1967, pp. 109—127.
19) M. O. Dayhoff, "Atlas of Protein Sequence and Structure," Vol. 5, National Biomedical Research Foundation, Washington, DC, 1978, p. 542.
20) J. L. Gallarda, K. L. Gleason, and K. L. Knight, *J. Immunol.*, 135, 4222—4228 (1985).
21) G. R. Dreesman and A. A. Benedict, *Proc. Natl. Acad. Sci. U.S.A.*, 54, 822—830 (1965).
22) E. Day, "Advanced Immunochemistry," John Wiley and Sons, New York, 1990, pp. 3—277.
23) C. A. Hasemann and J. D. Capra, in "Fundamental Immunology," 2nd Ed., ed. by W. E. Paul, Raven Press Ltd., New York, 1989, pp. 209—233.
24) R. Duvoisin, O. Heidmann, and J.-C. Jaton, *J. Immunol.*, 136, 4297—4302 (1986).
25) Y. Ashikari, Y. Arata, and K. Hamaguchi, *J. Biochem.*, 97, 517—528 (1985).
26) I. Pilz, E. Schwarz, and W. Palm, *Eur. J. Biochem.*, 75, 195—199 (1977).
27) H. Ohtani, K. Matsumoto, A. Saeki, and A. Hosono, *Lebensm. Wiss. Technol.*, 24, 152—158 (1991).